ANALOGS OF CATIONIC PROTEINS

This application is a divisional of application
Serial No 09/742,600, filed December 19, 2000, now
5 pending, which is a divisional of application Serial
No.09/214,214, filed December 23, 1998, which is a 371
National Stage Filing of PCT/US97/12609, filed July 17,
1997, now Patent No. 6,211,150, which is a
continuation-in-part of U.S. Application Serial No.
10 08/684,353, filed July 19, 1996, now abandoned, which
are incorporated by reference herein.

FIELD OF THE INVENTION

15   This invention relates generally to polypeptide
analogs of therapeutically active cationic proteins,
including but not limited to analogs of the
neurotrophic factors known as neurotrophin-3 (NT-3) and
brain-derived neurotrophic factor (BDNF). More
20 specifically, the invention relates to positively
charged polypeptides in which modifications have been
made in the native sequence, such that the analogs
possess lower isoelectric points and, concomitantly,
longer circulation times and/or improved absorption *in
25 vivo* following parenteral administration. The
invention also relates to materials and methods for the
recombinant production of such polypeptide analogs, to
antibodies thereof, and to pharmaceutical compositions
containing the analogs which can be used for the
30 treatment of various diseases and disorders.

BACKGROUND OF THE INVENTION

Following the administration of a therapeutic
35 protein by parenteral means, such as by subcutaneous,
intravenous or intramuscular injection, the
pharmacokinetic properties such as bioavailability,

circulation time and clearance rate can vary widely
from protein to protein. Even though there are active
efforts in many laboratories to develop alternative
routes of administration for protein products, little

5   is known about the factors that govern the
pharmacokinetic behavior of protein therapeutics
following such parenteral administration. It has been
shown that an increase in the molecular weight of a
protein can result in a preferential uptake by the

10   lymphatic system rather than the blood capillaries;
Supersaxo et al., Pharmaceutical Res., Volume 7, page
167 et seq. (1990). The molecular size of the
therapeutic protein also plays a key role in insulin
uptake, where dissociation of a zinc-induced hexamer to

15   monomeric form has been shown to be the rate-limiting
step in insulin absorbance; Kang et al., Diabetes Care,
Volume 14, pages 942-948 (1991). The clinical testing
in diabetic patients of monomeric insulin analogs, in
which the hexamer association site has been eliminated,

20   has demonstrated a more rapid uptake, leading to
significant improvements in glucose control in diabetic
patients; see Brange et al., Nature, Volume 333, page
679 et seq. (1988).

25   SUMMARY OF THE INVENTION

To assess the impact of the isoelectric point (pI)
on the pharmacokinetic behavior of proteins, certain
analogs of NT-3 and BDNF, in particular, have been

30   produced which have a relatively lower pI, yet retain
the structure and biological activity of the protein in
its "native" state (i.e., the protein of naturally
occurring amino acid sequence, as well as the met^{-1}
version thereof, both of which are referred to herein

35   as "wild type"). From these studies, it has now been
discovered that protein analogs engineered to possess a

lower pI and/or lower charge under physiological conditions than the wild type molecule, can also display longer *in vivo* circulation times (i.e., "half life") and improved absorption following administration

5   by injection. Although the invention is illustrated in this description with particular reference to human NT-3 and BDNF, it has broader applicability to any cationic proteins, and particularly basic proteins which in their native sequence have a pI greater than

10   about 7.0.

     It should be noted that the terms "protein" and "polypeptide" are used interchangeably throughout this description to mean one and the same thing.

15

     Briefly stated, the present invention is concerned with substitution, insertion and deletion analogs of cationic therapeutic proteins, and/or chemically modified versions of such therapeutic proteins, that

20   are characterized by a lower pI while also exhibiting longer circulation times and/or higher absorption relative to the unmodified proteins (i.e., of native sequence). The analog proteins of this invention are typically human therapeutic proteins which are usually,

25   but not necessarily, basic proteins. Preferably, these proteins also have a lower charge under physiological conditions compared to the unmodified basic protein.

     The present invention also concerns materials and

30   methods for the recombinant production of such analogs (as a preferred practical method), as well as to antibodies raised against the protein analogs, and to pharmaceutical compositions containing the analogs as biologically active agents for use in the treatment of

35   diseases and physical disorders.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the amino acid sequence for wild type human NT-3 (i.e., of "native" sequence) which has been produced recombinantly in *E. coli* bacterial cells and expressed with a methionine residue present at the N-terminus, i.e., r-metHuNT-3 (SEQ ID NO: 1). In the Figure, the amino acid numbering begins with the first residue after the initial methionine (Met^{-1}), since the naturally occurring protein is normally expressed in mammalian cells without the methionine residue. (Note that Sequence Listing herein counts Met residue as +1).

FIGURE 2 shows the nucleic acid sequence (SEQ ID NO: 2, and Fig. 2A) and amino acid sequence (SEQ ID NO: 3, and Fig. 2B) of a polypeptide analog of r-metHuNT-3 of the present invention, namely, NT-3$_{(1-119)}$R61A,K64D (with amino acid numbering in the Figure beginning with first residue after initial methionine).

FIGURE 3 shows the nucleic acid sequence (SEQ ID NO: 4, and Fig. 3A) and amino acid sequence (SEQ ID NO: 5, and Fig. 3B) of another polypeptide analog of r-metHuNT-3 according to the present invention, namely, NT-3$_{(1-117)}$R61A,K64D (amino acid numbering in Figure again beginning with first residue after initial methionine).

FIGURE 4 shows the ELISA assay calibration (standard) curves for wild type NT-3 (i.e., having SEQ ID NO: 1) and for the NT-3 analogs of SEQ ID NOS: 3 and 5. The optical density is plotted against sample concentration in nanograms per milliliter (ng/ml). The cross-reactivity to NT-3 analogs in the ELISA assay is

approximately ten percent. Serum samples from
pharmacokinetic studies were analyzed by ELISA, and
concentrations of wild type NT-3 and NT-3 analogs were
determined by calibration against the appropriate
5   standard curve.

        FIGURE 5 depicts a size exclusion high performance
liquid chromatogram (SEC-HPLC) in which wild type NT-3
is compared to the NT-3 analogs of Figures 2 and 3.
10 Units of absorbance ("AU") are plotted on the vertical
axis, and the time of elution to the peak (in minutes)
is shown on the horizontal axis. As shown, the analogs
co-elute with the wild type molecule, demonstrating
that the noncovalent dimer structure of wild type NT-3
15 has not been disrupted in either of the analogs. No
aggregated protein was detected in any of these
preparations. Figure legends: (_____) wild type NT-3;
(----) NT-3$_{(1-117)}$R61A,K64D; and (__.__)
NT-3$_{(1-119)}$R61A,K64D.
20

        FIGURE 6 depicts a cation exchange high
performance liquid chromatogram (CEX-HPLC) in which
wild type NT-3 is compared to the NT-3 analogs of
Figures 2 and 3. "AU" (vertical axis) indicates
25 absorbance units. Time of elution to peak is shown on
the horizontal axis. In this figure the analogs elute
sooner than wild type NT-3, which is consistent with
the lower isoelectric points of the analogs. Figure
legends: (_____) wild type NT-3; (----) NT-3$_{(1-117)}$R61A,K64D; and (__.__) NT-3$_{(1-119)}$R61A,K64D.
30 117)R61A,K64D; and (__.__) NT-3$_{(1-119)}$R61A,K64D.

        FIGURE 7 depicts a silver stained SDS
polyacrylamide gel electrophoresis (SDS-PAGE)
chromatogram in which wild type NT-3 (see Figure 1) is
35 compared to the NT-3 analogs of Figures 2 and 3. All
three samples run as a single band with approximately

the same molecular weight of the monomeric form. None
of the samples are seen to contain significant amounts
of higher molecular weight oligomers or lower molecular
weight fragments. Lane 1: NT-3$_{(1-117)}$R61A,K64D, 2.5 µg;
5   Lane 2: NT-3$_{(1-119)}$R61A,K64D, 2.5 µg; Lane 3; wild type
NT-3, 2.5 µg; Lane 4: wild type NT-3, 12.5 µg; Lane 5:
molecular weight markers.

     FIGURE 8 shows the serum concentration (in
10   nanograms per milliliter) versus time(in hours)
profiles for the proteins of SEQ ID NOS: 1, 3 and 5
after intravenous (IV) administration to test rats.
The dose level was 1 milligram per kilogram (mg/kg) of
body weight. The concentration profiles are biphasic.
15   The initial distribution phase was followed by a slower
elimination phase. Each point on the graph represents
an average of three animals.

     FIGURE 9 shows the serum concentration (in
20   nanograms per milliliter) versus time (in hours) curves
in rats for the proteins of SEQ ID NOS: 1, 3 and 5
following subcutaneous (SC) administration of 1 mg/kg,
with each point on the graph again representing an
average of three animals. The absorption phase is
25   characterized by an increase in serum concentration to
a peak. Wild type NT-3 (SEQ ID NO: 1) showed the most
rapid decline after attaining maximal concentration.

                DETAILED DESCRIPTION OF THE INVENTION
30

     The principles of this invention have broad
applicability to any cationic proteins for which a
reduction in the pI and, optionally, protein charge
will result in an enhancement of therapeutically
35   relevant biological properties such as circulation time
and/or absorption following parenteral administration.

By way of illustration, such proteins include but are
not limited to basic proteins such as NT-3, BDNF,
macrophage growth and differentiation factor and
various known isoforms thereof having essentially the
5   same ability to increase blood platelet production *in
vivo* and *ex vivo* (referred to herein collectively as
"MGDF"), and keratinocyte growth factor (KGF).
Detailed descriptions of these factors, their
biological properties, and methods for their
10   preparation and testing are given in the patent
literature: NT-3 in published PCT application
WO 91/03569; BDNF in United States patents
No.5,180,820, No.5,229,500, No.5,438,121 and No.
5,453,361, and in published PCT application WO
15   91/03568; MGDF in published PCT applications WO
95/26745, WO 95/21919, and WO 95/21920; and KGF in
published PCT application WO 90/08771.

In essence, the objective of this invention is to
20   make one or more modifications to the primary structure
of the wild type protein that preserve the protein
structure and biological activity of the protein, but
which also results in a lower isoelectric point and,
preferably, a lower charge at physiological pH. The
25   particular way in which these modifications are made is
not critical, and any procedure can be used which
effects the aforementioned changes to achieve the
described enhancements in properties. Merely by way of
illustration, appropriate modifications can be
30   accomplished by use of site directed mutagenesis
involving the addition of acidic residues to the
sequence by insertion and/or replacement mutations,
and/or removal of basic residues by deletion and/or
replacement mutations. Alternatively, chemical groups
35   or moieties can be added to selected sites (i.e., on
amino acid residues) in the protein chain of the wild

type molecule to accomplish the same end purpose (i.e.,
reduction of pI and charge with preservation of
structure and biological activity). A specific example
is the succinylation of selected residues in the
5   protein chain.

Nucleic acids which encode protein analogs in
accordance with this invention (i.e., wherein one or
more amino acids are designed to differ from the wild
10   type polypeptide) may be produced using site directed
mutagenesis or PCR amplification in which the primer(s)
have the desired point mutations. For a detailed
description of suitable mutagenesis techniques, see
Sambrook et al., Molecular Cloning: A Laboratory
15   Manual, Cold Spring Harbor Laboratory Press, Cold
Spring Harbor, NY (1989) and/or Ausubel et al.,
editors, Current Protocols in Molecular Biology, Green
Publishers Inc. and Wiley and Sons, NY (1994).
Chemical synthesis using methods described by Engels et
20   al. in Angew. Chem. Intl. Ed., Volume 28, pages 716-734
(1989), may also be used to prepare such nucleic acids.

The DNA molecules may be used to express the
analog polypeptides of the invention by recombinant
25   methods familiar to those skilled in the art, including
but not limited to methods described in the above
mentioned patents or patent applications for NT-3,
BDNF, KGF and MGDF. By way of illustration, a nucleic
acid sequence encoding an analog polypeptide of this
30   invention is inserted into an appropriate biologically
functional vector (e.g., circular plasmid or viral DNA)
for expression in a suitable host cell . The vector
includes regulatory sequences for expression of the
inserted nucleic acid sequence and is selected to be
35   functional in the particular host cell employed (i.e.,
the vector is compatible with the host cell machinery,

such that amplification and/or expression of the gene can occur). The polypeptide may be amplified/expressed in prokaryotic, yeast, insect (baculovirus systems) and/or eukaryotic host cells. Selection of the host

5   cell will depend at least in part on whether the polypeptide expression product is to be glycosylated. If glycosylation is desired, then yeast, insect or mammalian host cells are preferred for use.

10   Typically, the vectors will contain a 5' flanking sequence (also referred to as a "promoter") and other regulatory elements, as well as enhancer(s), an origin of replication element, a transcriptional termination element, a complete intron sequence containing a donor

15   and acceptor splice site, a signal peptide sequence, a ribosome binding site element, a polyadenylation sequence, a polylinker region for inserting the nucleic acid encoding the polypeptide to be expressed, and a selectable marker element.

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     The 5' flanking sequence may be the innate 5' flanking sequence from the wild type gene, or it may be homologous (i.e., from the same species and/or strain as the host cell), heterologous (i.e., from a species

25   other than the host cell species or strain), hybrid (i.e., a combination of 5' flanking sequences from more than one source), or synthetic. The source of the 5' flanking sequence may be any unicellular prokaryotic or eukaryotic organism, any vertebrate or invertebrate

30   organism, or any plant, provided that the 5' flanking sequence is functional in, and can be activated by, the host cell machinery.

     The origin of replication element is typically a

35   part of prokaryotic expression vectors purchased commercially, and aids in the amplification of the

vector in a host cell. Amplification of the vector to a certain copy number can, in some cases, be important for optimal expression of the polypeptide. If the vector of choice does not contain an origin of

5   replication site, one may be chemically synthesized based on a known sequence and then ligated into the vector.

The transcription termination element is typically

10   located 3' to the end of the polypeptide coding sequence and serves to terminate transcription of the polypeptide. Usually, the transcription termination element in prokaryotic cells is a G-C rich fragment followed by a poly-T sequence. While the element is

15   easily cloned from a library or even purchased commercially as part of a vector, it can also be readily synthesized using methods for nucleic acid synthesis such as those referred to above.

20   A selectable marker gene element encodes a protein necessary for the survival and growth of a host cell grown in a selective culture medium. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin,

25   tetracycline, or kanamycin for prokaryotic host cells, (b) complement auxotrophic deficiencies of the cell; or (c) supply critical nutrients not available from complex media. Preferred selectable markers are the kanamycin resistance gene, the ampicillin resistance

30   gene, and the tetracycline resistance gene.

The ribosome binding element, commonly called the Shine-Dalgarno sequence (for prokaryotes) or the Kozak sequence (for eukaryotes), is necessary for the

35   initiation of translation for mRNA. The element is typically located 3' to the promoter and 5' to the

coding sequence of the polypeptide to be synthesized.
The Shine-Dalgarno sequence is varied but is typically
a polypurine (i.e., having a high A-G content). Many
Shine-Dalgarno sequences have been identified, each of
5 which can be readily synthesized using methods set
forth above and used in a prokaryotic vector.

In those cases where it is desirable for the
polypeptide to be secreted from the host cell, a signal
10 sequence may be used to direct the polypeptide out of
the host cell where it is synthesized. Typically, the
signal sequence is positioned in the coding region of
nucleic acid sequence, or directly at the 5' end of the
coding region. Many signal sequences have been
15 identified, and any of them that are functional in the
selected host cell may be used here. Consequently, the
signal sequence may be homologous or heterologous to
the polypeptide. Additionally, the signal sequence may
be chemically synthesized using methods such as those
20 referred to set above.

Host cells may be prokaryotic host cells (such as
E. coli) or eukaryotic host cells (such as yeast,
insect or vertebrate cells). The host cell, when
25 cultured under suitable nutrient conditions, can
synthesize the polypeptide, which can subsequently be
collected by isolation from the culture medium (if the
host cell secretes it into the medium) or directly from
the host cell producing it (if not secreted). After
30 collection, the polypeptide can be purified using
methods such as molecular sieve chromatography,
affinity chromatography, and the like. In general, if
the polypeptide is expressed in E. coli it will contain
a methionine residue at the N-terminus in its recovered
35 form (i.e. met^{-1}), unless expressed in a strain of E.

coli in which the methionine is enzymatically cleaved
off by the host.

Suitable cells or cell lines may also be mammalian
cells, such as Chinese hamster ovary cells (CHO) or 3T3
cells. The selection of suitable mammalian host cells
and methods for transformation, culture, amplification,
screening and product production and purification are
known in the art. Other suitable mammalian cell lines
are the monkey COS-1 and COS-7 cell lines, and the CV-1
cell line. Further exemplary mammalian host cells
include primate cell lines and rodent cell lines,
including transformed cell lines. Normal diploid
cells, cell strains derived from *in vitro* culture of
primary tissue, as well as primary explants, are also
suitable. Candidate cells may be genotypically
deficient in the selection gene, or they may contain a
dominantly acting selection gene. Still other suitable
mammalian cell lines include but are not limited to,
HeLa, mouse L-929 cells, 3T3 lines derived from Swiss,
Balb-c or NIH mice, BHK or HaK hamster cell lines.

Insertion (also referred to as "transformation" or
"transfection") of the vector into the selected host
cell may be accomplished using calcium chloride,
electroporation, microinjection, lipofection or the
DEAE-dextran method. The method selected will in part
be a function of the type of host cell to be used.
These methods and other suitable methods are well known
to the skilled artisan, and are set forth, for example,
in Sambrook et al., above.

The host cells containing the vector may be
cultured using standard media well known to the skilled
artisan. The media will usually contain all of the
nutrients necessary for the growth and survival of the

cells. Suitable media for culturing *E. coli* cells are, for example, Luria Broth (LB) and/or Terrific Broth (TB). Suitable media for culturing eukaryotic cells are RPMI 1640, MEM, DMEM, all of which may be

5   supplemented with serum and/or growth factors as required by the particular cell line being cultured. A suitable medium for insect cultures is Grace's medium supplemented with yeastolate, lactalbumin hydrolysate and/or fetal calf serum as necessary.

10

Typically, an antibiotic or other compound useful for selective growth of the transformed cells is added as a supplement to the media. The compound to be used will be dictated by the selectable marker element

15   present on the plasmid with which the host cell was transformed. For example, where the selectable marker element is kanamycin resistance, the compound added to the culture medium will be kanamycin.

20   The amount of polypeptide produced in the host cell can be evaluated using standard methods known in the art. Such methods include, without limitation, Western blot analysis, SDS-polyacrylamide gel electrophoresis, non-denaturing gel electrophoresis,

25   HPLC separation, immunoprecipitation, and/or activity assays such as DNA binding gel shift assays.

If the polypeptide has been designed to be secreted from the host cells, the majority of

30   polypeptide will likely be found in the cell culture medium. If, however, the polypeptide is not secreted, it will be present in the cytoplasm (for eukaryotic, Gram-positive bacteria, and insect host cells) or in the periplasm (for Gram-negative bacteria host cells).

35

For intracellular polypeptide, the host cells are typically first disrupted mechanically or osmotically to release the cytoplasmic contents into a buffered solution. The polypeptide is then isolated from this

5   solution. Purification of the polypeptide from solution can thereafter be accomplished using a variety of techniques. If the polypeptide has been synthesized such that it contains a tag such as Hexahistidine or other small peptide at either its carboxyl or amino

10   terminus, it may be purified in a one-step process by passing the solution through an affinity column where the column matrix has a high affinity for the tag or for the polypeptide directly (i.e., a monoclonal antibody). For example, polyhistidine binds with great

15   affinity and specificity to nickel, thus an affinity column of nickel (such as the Qiagen nickel columns) can be used for purification. (See, for example, Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1994).

20

Where, on the other hand, the polypeptide has no tag and no antibodies are available, other well known procedures for purification can be used. Such procedures include, without limitation, ion exchange

25   chromatography, molecular sieve chromatography, HPLC, native gel electrophoresis in combination with gel elution, and preparative isoelectric focusing ("Isoprime" machine/technique, Hoefer Scientific). In some cases, two or more of these techniques may be

30   combined to achieve increased purity.

If it is anticipated that the polypeptide will be found primarily in the periplasmic space of the bacteria or the cytoplasm of eukaryotic cells, the

35   contents of the periplasm or cytoplasm, including inclusion bodies (e.g., Gram-negative bacteria) if the

processed polypeptide has formed such complexes, can be extracted from the host cell using any standard technique known to the skilled artisan. For example, the host cells can be lysed to release the contents of
5 the periplasm by the use of a French press, homogenization, and/or sonication. The homogenate can then be centrifuged.

In addition to preparing the polypeptide analogs
10 of this invention by recombinant DNA techniques, the polypeptides may be prepared by chemical synthesis methods (such as solid phase peptide synthesis) using techniques known in the art, including those set forth by Merrifield et al. in J. Am. Chem. Soc., Volume 85,
15 page 2149 (1964), by Houghten et al. in Proc. Natl. Acad. Sci. USA, Volume 82, page 5132 (1985), and by Stewart and Young in Solid Phase Peptide Synthesis, Pierce Chem. Co, Rockford, IL (1984). Chemically synthesized polypeptides may be oxidized using methods
20 set forth in these references to form disulfide bridges.

The pI and charge of the protein analogs resulting from any of the aforementioned methods can be measured
25 using standard techniques, such as those described further below in conjunction with the specific embodiments.

Chemically modified polypeptide compositions
30 (i.e., "derivatives") where the polypeptide is linked to a polymer in order to modify properties are included within the scope of the present invention. The polymer is typically water soluble so that the protein to which it is attached does not precipitate in an aqueous
35 environment, such as a physiological environment. The polymer may have a single reactive group, such as an

active ester for acylation or an aldehyde for
alkylation, so that the degree of polymerization may be
controlled.   A preferred reactive aldehyde is
polyethylene glycol propionaldehyde, which is water

5   stable, or mono C1-C10 alkoxy or aryloxy derivatives
thereof (see U.S. Patent 5,252,714). The polymer may
be branched or unbranched. Preferably, for therapeutic
use of the end-product preparation, the polymer will be
pharmaceutically acceptable. The water soluble

10   polymer, or mixture thereof if desired, may be selected
from the group consisting of, for example, polyethylene
glycol (PEG), monomethoxy-polyethylene glycol, dextran,
cellulose, or other carbohydrate based polymers, poly-
(N-vinyl pyrrolidone) polyethylene glycol, propylene

15   glycol homopolymers, a polypropylene oxide/ethylene
oxide co-polymer, polyoxyethylated polyols (e.g.,
glycerol) and polyvinyl alcohol.

In general, the polypeptide analogs of this
20   invention will be useful for the same purposes for
which the wild type proteins from which they are
derived are known to be useful. For instance, NT-3 is
currently under clinical study for the treatment of
peripheral (including diabetic) neuropathies, while
25   BDNF is under clinical study for the treatment of
amyotrophic lateral sclerosis (ALS). KGF is known to
be active as a tissue growth and repair factor, and is
currently in human clinical development for the
treatment of chemotherapy- or radiation-induced
30   mucositis. MGDF (in the form of a pegylated
derivative) is in clinical development for the
stimulation of platelet production as an adjunct to
chemotherapy-induced thrombocytopenia. However, it is
expected that the analogs of this invention will offer
35   advantages over the unmodified forms from the

standpoint of enhanced therapeutic half life and
absorbability.

For therapeutic purposes, the analog polypeptides
5   of this invention will typically be formulated into
suitable pharmaceutical compositions adapted for
therapeutic delivery, which constitutes an additional
aspect of this invention. Such pharmaceutical
compositions will typically comprise a therapeutically
10   active amount of an analog polypeptide, alone or
together with one or more excipients, carriers, or
other standard ingredients for a pharmaceutical
composition. The carrier material may be water for
injection, preferably supplemented with other materials
15   common in solutions for administration to mammals.
Typically, the analog polypeptide will be administered
in the form of a composition comprising a purified form
of the polypeptide (which may be chemically modified)
in conjunction with one or more physiologically
20   acceptable carriers, excipients, or diluents. Neutral
buffered saline or saline mixed with serum albumin are
exemplary appropriate carriers. Other standard
carriers, diluents, and excipients may be included as
desired.
25

The pharmaceutical compositions of this invention
may be prepared for storage by mixing the selected
composition having the desired degree of purity with
optional physiologically acceptable carriers,
30   excipients, or stabilizers (Remington's Pharmaceutical
Sciences, 18th edition, A.R. Gennaro, ed., Mack
Publishing Company, 1990) in the form of a lyophilized
cake or an aqueous solution. Acceptable carriers,
excipients or stabilizers are nontoxic to recipients
35   and are preferably inert at the dosages and
concentrations employed, and include buffers such as

phosphate, citrate, acetate, succinate or other organic
acid salts; antioxidants such as ascorbic acid; low
molecular weight polypeptides; proteins, such as serum
albumin, gelatin, or immunoglobulins; hydrophilic
5   polymers such as polyvinylpyrrolidone; amino acids such
as glycine, glutamine, asparagine, arginine or lysine;
monosaccharides, disaccharides, and other carbohydrates
including glucose, mannose, or dextrins; chelating
agents such as EDTA; sugar alcohols such as mannitol or
10 sorbitol; salt-forming counterions such as sodium;
and/or nonionic surfactants such as Tween, Pluronics or
polyethylene glycol (PEG).

        Any composition of this invention which is
15 intended to be used for *in vivo* administration must be
sterile. Sterilization is readily accomplished by
filtration through sterile filtration membranes. Where
the composition is lyophilized, sterilization using
these methods may be conducted either prior to, or
20 following, lyophilization and reconstitution. The
composition for parenteral administration ordinarily
will be stored in lyophilized form or in solution.

        The amount of polypeptide that will be effective
25 in the treatment of a particular disorder or condition
will depend on the nature of the polypeptide and
disorder or condition, as well as the age and general
health of the recipient, and can be determined by
standard clinical procedures. Where possible, it will
30 be desirable to determine the dose-response curve of
the pharmaceutical composition first *in vitro*, as in
bioassay systems, and then in useful animal model
systems *in vivo* prior to testing in humans. In
general, suitable *in vivo* amounts can be developed
35 based on a knowledge of the therapeutically effective
doses known for the wild type protein on which the

analogs are based. The skilled practitioner,
considering the therapeutic context, type of disorder
under treatment, etc., will be able to ascertain proper
dosing without undue effort.

5

Methods of introduction for administration
purposes include intradermal, intramuscular,
intraperitoneal, intravenous, and subcutaneous. In
addition, the invention also encompasses pharmaceutical

10   compositions comprising the polypeptide analogs
administered via liposomes, microparticles or
microcapsules, which may be particularly useful to
achieve sustained release.

15   Special delivery devices may needed in the case of
some of the polypeptide analogs, such as those of NT-3,
BDNF and other neurotrophic factors intended for the
treatment of neurological conditions associated with
the brain and other areas of the central nervous

20   system. Such devices may include implants and osmotic
pumps for intrathecal and intracranial delivery, for
instance.

The analogs of this invention can also be used in
25   accordance with standard procedures to generate
antibodies that are useful for medically related
purposes, such as for the monitoring of blood levels of
the corresponding analog in a subject undergoing
therapeutic treatment. Various procedures known in the

30   art can be employed for the production of polyclonal
antibodies that recognize epitopes of the polypeptides.
For the production of antibody, various host animals
can be immunized by injection with an analog
polypeptide, or fragment or derivative thereof,

35   including but not limited to rabbits, mice, rats, etc.
Various adjuvants may be used to increase the

immunological response, depending on the host species, including but not limited to Freund's, mineral gels such as aluminum hydroxide (alum), surface active substances such as lysolecithin, pluronic polyols,
5   polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as Bacille Calmette-Guerin and *Corynebacterium parvum*.

10   For the preparation of monoclonal antibodies directed toward the analog polypeptides, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed
15   by Kohler and Milstein and described in Nature, Volume 256, pages 495-497 (1975), as well as the trioma technique, the human B-cell hybridoma technique described by Kozbor et al in Immunology Today, Volume 4, page 72 (1983), and the EBV-hybridoma technique to
20   produce monoclonal antibodies described by Cole et al in "Monoclonal Antibodies and Cancer Therapy", Alan R. Liss, Inc., pages 77-96 (1985), are all useful for preparation of monoclonal antibodies.

25   In addition, a molecular clone of an antibody to an epitope or epitopes of the polypeptide can be prepared with known techniques. In particular, recombinant DNA methodology may be used to construct nucleic acid sequences which encode a monoclonal
30   antibody molecule or antigen-binding region thereof; see, for example, Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982).

35   The antibodies are useful for both *in vivo* and *in vitro* diagnostic purposes, particularly in labeled form

to detect the presence of the polypeptides in a fluid
or tissue sample.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The invention is described in further detail with
reference to the following materials, methods,
procedures and test results. Amino acid residues of
proteins are identified the in conventional manner
using the established three letter designations (for
example, "met" for methionine, "val" for valine, etc.)
or in some cases the established single letter
designations (for example, "M" for methionine, "R" for
arginine, etc.) throughout the text.

Materials and Methods:

Preparation of NT-3 analogs. Amino acid residues for
substitution in the native sequence of human NT-3 were
selected with the aim of preserving the core structure
and biological/therapeutic activity (see Holland et
al., J. Mol. Biol., Volume 239, pages 385-400, 1994;
Ibanez et al., in Cell, Volume 69, pages 329-341, 1992
and also in EMBO Journal, Volume 12, pages 2281-2293,
1993). The actual substitutions that were made in the
native sequence of human NT-3 are reflected in the
sequences shown in Figures 2 and 3, respectively. In
one analog, shown in Figure 2, the following two
substitutions were made: arginine at position 61
(arg$_{61}$) was replaced by alanine (ala), and lysine at
position 64 (lys$_{64}$) was replaced by aspartic acid
(asp). This analog was designated "NT-3$_{(1-119)}$
R61A,K64D". A second analog, shown in Figure 3, had
these same two amino acid substitutions and, in
addition, was truncated at residue 117 (thus deleting
arg$_{118}$ and thr$_{119}$). This analog was designated "NT-3$_{(1-}$

117)R61A,K64D". To create these analogs, the mutations
were introduced in the sequence of human NT-3 by
standard Polymerase Chain Reaction (PCR) technology.
For NT-3(1-119)R61A,K64D, chemically synthesized
5   oligonucleotides were used in pairs to create fragments
of the NT-3 gene comprising the front portion up to the
site of the mutations at the codons corresponding to
positions 61 and 64 and the back portion of the gene
from the mutant codons to the end. A second PCR was
10   carried out combining the front and back portions to
create the full length nucleic acid molecule encoding
the two mutations at positions 61 and 64, respectively.
For NT-3(1-117)R61A,K64D, the foregoing procedure was
repeated, except the back portion omitted the codons
15   for arginine and threonine at positions 118 and 119.

Expression in E. coli. To express the analogs in
E. coli, a DNA sequence encoding for a methionine
residue was included at the 5' end and a stop codon was
20   placed at the 3' end in each case. In addition,
cutting sites for the restriction enzymes XbaI and
HindIII were placed at the extreme 5' and 3' ends of
the gene, respectively, and a synthetic ribosome
binding site was placed an appropriate distance
25   upstream of the initiating methionine. The resulting
synthetic gene fragments, flanked by XbaI and HindIII
restriction sites at the 5' and 3' ends, respectively,
contained a ribosome binding site, the ATG start codon
(encoding methionine), the sequence encoding the
30   analog, and a stop codon. The fragments were digested
with restriction endonucleases NdeI and BamHI, and then
ligated into the vector pAMG12.

The expression plasmid pAMG12 can be derived from
35   the plasmid pCFM1656 (ATCC Accession No. 69576,
deposited February 24, 1994) by making a series of site

directed base changes by PCR overlapping oligo
mutagenesis and DNA sequence substitutions. Starting
with the BglII site (plasmid base pair no. 180)
immediately 5' to the plasmid replication promoter
P$_{copB}$ and proceeding toward the plasmid replication
genes, the base pair (bp) changes are as follows:

pAMG12 bp no.	bp in pCFM1656 pAMG12	bp changed to in pAMG12
# 204	T/A	C/G
# 428	A/T	G/C
# 509	G/C	A/T
# 617	– –	insert two G/C bp
# 679	G/C	T/A
# 980	T/A	C/G
# 994	G/C	A/T
# 1004	A/T	C/G
# 1007	C/G	T/A
# 1028	A/T	T/A
# 1047	C/G	T/A
# 1178	G/C	T/A
# 1466	G/C	T/A
# 2028	G/C	bp deletion
# 2187	C/G	T/A
# 2480	A/T	T/A
# 2499-2502	AGTG TCAC	GTCA CAGT
# 2642	TCCGAGC AGGCTCG	bp deletion
# 3435	G/C	A/T
# 3446	G/C	A/T
# 3643	A/T	T/A

In addition, the DNA sequence between the unique AatII (position #4364 in pCFM1656) and SacII (position #4585 in pCFM1656) restriction sites is substituted with the following DNA sequence:

[AatII sticky end]

```
5'       CGTAACGTATGCATGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAA-
3' GCACGCATTGCATACGTACCAGAGGGGTACGCTCTCATCCCTTGACGGTCCGTAGTT-

-TAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTG-
-ATTTTGCTTTCCGAGTCAGCTTTCTGACCCGGAAAGCAAAATAGACAACAAACAGCCAC-

-ACGCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGG-
-TGCGAGAGGACTCATCCTGTTTAGGCGGCCCTCGCCTAAACTTGCAACGCTTCGTTGCC-

-CCGGAGGGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAG-
-GGCCTCCCACCGCCCGTCCTGCGGGCGGTATTTGACGGTCCGTAGTTTAATTCGTCTTC-

-CCATCCTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTTTGTTTATTTTTCTAAAT-

-CGGTAGGACTGCCTACCGGAAAAACGCAAAGATGTTTGAGAAAACAAATAAAAAGATTTA-
                    AatII
-ACATTCAAATATGGACGTCTCATAATTTTTAAAAAATTCATTTGACAAATGCTAAAATTC-
-TGTAAGTTTATACCTGCAGAGTATTAAAAATTTTTTAAGTAAACTGTTTACGATTTTAAG-

-TTGATTAATATTCTCAATTGTGAGCGCTCACAATTTATCGATTTGATTCTAGATTTGAGT-
-AACTAATTATAAGAGTTAACACTCGCGAGTGTTAAATAGCTAAACTAAGATCTAAACTCA-

-TTTAACTTTTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGAGCTCACTAGT-
-AAATTGAAAATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGCTCGAGTGATCA-
                    SacII
-GTCGACCTGCAGGGTACCATGGAAGCTTACTCGAGGATCCGCGGAAAGAAGAAGAAGAAG-
-CAGCTGGACGTCCCATGGTACCTTCGAATGAGCTCCTAGGCGCCTTTCTTCTTCTTCTTC-

-AAGAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAA-
-TTCTTTCGGGCTTTCCTTCGACTCAACCGACGACGGTGGCGACTCGTTATTGATCGTATT-

-CCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACCGCTCTT-
-GGGGAACCCCGGAGATTTGCCCAGAACTCCCCAAAAAACGACTTTCCTCCTTGGCGAGAA-

-CACGCTCTTCACGC 3'
-GTGCGAGAAGTG    5'
```

[SacII sticky end]

The ligation product was transformed into competent host cells of *E. coli* strain FM15. Resulting colonies were screened for the production of recombinant protein and those colonies producing the correct-sized protein were verified by DNA sequencing. The correct strain was inoculated for fermentation by

transferring a small amount to Luria Broth (10 g/l of Trypticase-Peptone, 10 g/l of Yeast Extract, and 5 g/l of sodium chloride) and incubating at 30°C for sixteen hours with stirring at 250 rpm. The culture was

5   transferred to sterile medium that had been sterilized in place in a fermentor, then the mass of the cells was increased using continuous feeds of glucose and organic nitrogen, before being induced with lactose. After induction, the fermentation was halted, the cells were

10   harvested by centrifugation, the supernatant was removed, and the remaining cell paste was frozen.

Protein purification. Cells from the paste were broken by high pressure homogenization and inclusion bodies

15   were recovered by centrifugation. The inclusion bodies were solubilized in guanidine-HCl, then diluted into urea. After standing for several days, the solution was adjusted to pH 3, diluted with water, centrifuged, and eluted in series through cation exchange and

20   hydrophobic interaction chromatography columns. The peak fractions were pooled and sterile filtered.

Isoelectric point and protein charge. The isoelectric points of wild type human NT-3 (r-metHuNT-3, SEQ ID

25   NO:1), and of the analogs thereof (i.e., SEQ ID NOS: 3 and 5) were calculated using the "GCG" protein/DNA Sequence Analysis Software Package available from Genetics Computer Group, Inc., Madison, Wisconsin. The charge of the molecule at physiological pH (assumed to

30   be pH 7.4) was estimated using the same software. The pI of the first analog, NT-3$_{(1-119)}$R61A,K64D (SEQ ID NO: 3), was calculated to be about 0.9 pH units below that of wild type NT-3 (8.5, compared to 9.4). The charge at physiologic pH (7.4) for this analog

35   represented a reduction of about 2.5 pH units from that of wild type NT-3 (i.e., from approximately +7 to

+4.5). The pI for the other analog, NT-3$_{(1-117)}$R61A,K64D (SEQ ID NO: 5) was calculated to be approximately 8.2, which was about 1.2 pH units lower than the pI of wild type NT-3 (9.4). Moreover, the

5   charge at physiologic pH for this analog was decreased by approximately 3.5 pH units, to about +3.5, relative to wild type NT-3 (+7).

ELISA Assay. The ELISA assay was conducted on 96-well

10   plates coated with a monoclonal antibody raised against human NT-3. A rabbit polyclonal antibody conjugated to horse radish peroxidase was used as the secondary antibody. Serum samples, calibration standards, and quality control samples were diluted with phosphate

15   buffer to a 50% serum matrix before assay. The sample size was 100 microliters (μl) per well. Each sample was assayed in duplicate. The limits of quantification were 0.65, 4.00, and 4.05 ng/ml of serum for wild type NT-3, NT-3$_{(1-119)}$R61A,K64D and NT-3$_{(1-117)}$R61A,K64D,

20   respectively.

Size Exclusion Chromatography (SEC-HPLC). Size exclusion chromatography on each sample was performed using a Waters 600 system in conjunction with a

25   G2000SWXL column (TosoHaas). Samples were eluted at a flow rate of 0.7 milliliters per minute (ml/min) in a buffer consisting of 100 mM sodium phosphate, 0.5 M NaCl, pH 6.09. Peaks were detected at a wavelength of 230 nanometers (nm).

30

Cation Exchange Chromatography (CEX-HPLC). CEX-HPLC
was performed using a Waters 625 system with a Resource
S column (Pharmacia, Uppsala, Sweden). Samples were
eluted at a flow rate of one milliliter per minute
5   using a sodium chloride gradient from 0 - 1 M in 20 mM
Tris HCl, pH 8.5. Peaks were detected using a
wavelength of 220 nm.

Silver Stained SDS-PAGE. Proteins were diluted with 2%
10   SDS, mixed with sample buffer, and heated for five
minutes in boiling water. The separation was conducted
according to manufacturer's instructions using precast
TRIS-Tricine gradient gels, 10-20%, from ISS
(Integrated Separations Systems, Natick, MA). Silver
15   staining was done according to the procedure of Blum et
al. in Electrophoresis, Volume 8, pages 93-99 (1987).

Mitogenic Bioassay With 3T3trkC Cells. The biological
activity of r-metHuNT-3 as a reference standard is
20   determined by means of a cell mitogenic bioassay
utilizing 3T3trkC cells. These cells are created by
transfecting 3T3 cells (ATCC), which normally do not
express trkC receptor on their surface, with plasmid
pcDNA1/neo (Invitrogen, San Diego, CA) modified to
25   contain the DNA sequence for human trkC receptor
protein. See Shelton et al., Journal of Neuroscience,
Volume 15, pages 477-491 (1995) for the sequence of the
trkC gene, and Valenzuela et al., Neuron, Volume 10,
pages 963-974 (1993) for an illustrative transfection
30   procedure. The transfected cells are maintained at 37
± 2°C, in a high humidity incubator under an atmosphere
containing 5.5 ± 1.0% CO_2 and in Dulbecco's Minimum
Essential Medium with fetal bovine serum and G-418
Sulfate. The cells are distributed into 96-well plates
35   for each assay. After approximately twenty four hours
of incubation time under the same conditions, the

maintenance medium is replaced with RPMI 1640 and test
samples are added. Varying concentrations of a
standard and a test sample of r-metHuNT-3 are prepared
in RPMI 1640 and added to the appropriate wells. The
5   plates are returned to the incubator for approximately
twenty four hours, then the cells are stained for
viability with 3-(4,5-dimethylthiazol-2-yl)-5-(3-
carboxymethoxyphenyl) -2-(4-sulfophenyl)2H-tetrazolium
salt (MTS), a tetrazolium compound. The plates are
10   returned to the incubator for approximately five hours,
after which the optical density of each well is read at
490 nm. A dose-response standard curve is constructed
and a linear regression analysis is performed on the
linear portion of the standard curve. The
15   concentration of test sample dilutions falling within
the linear extracted range of the standard curve are
then determined.

In Vivo Biological Testing. The effect of amino acid
20   changes on the biological properties of a molecule was
evaluated _in vivo_ in male Sprague Dawley rats, using a
"cross-over" test design. In particular, nine animals
were divided into three groups of three rats each, and
the rats were administered wild type NT-3, NT-3$_{(1-119)}$
25   R61A,K64D, or NT-3$_{(1-117)}$R61A,K64D at a dose of 1
milligram per kilogram of body weight (mg/kg) each.
The test material was administered either (1)
intravenously (IV) as a first dose, then subcutaneously
(SC) at twenty four hours after the first dose, or (2)
30   in the reverse manner. Serial blood samples were
collected before dosing and at 1, 5, 15, and 30
minutes, and 1, 2, 4, and 8 hours after an IV dose.
Following subcutaneous dosing, samples were collected
at 10 and 30 minutes, and at 1, 2, 4, and 8 hours.
35   Blood serum concentrations of NT-3 were determined
using an ELISA assay (see above). The antibodies used

in the assays were specific for quantitation of wild
type NT-3. Though not fully optimized, the antibodies
displayed sufficient cross-reactivity with NT-3$_{(1-119)}$R61A,K64D and NT-3$_{(1-117)}$ R61A,K64D to allow for
5   quantitation of these analogs also. Standard curves
were prepared for each protein (see Figure 4).

Test Results:

10   Characterization of NT-3 analogs. Both of the analogs,
NT-3$_{(1-119)}$R61A,K64D and NT-3$_{(1-117)}$ R61A,K64D, were seen
to retain the activity of wild-type NT-3 (r-metHuNT-3)
in the PC-12 in vitro bioassay (Table 1). In fact, the
bioactivity of the analogs as evaluated in this assay
15   appeared to be somewhat greater than that of wild-type
NT-3, perhaps indicating an increased affinity for the
trkC (NT-3) receptor.

Table 1. In Vitro Bioactivity of NT-3 Proteins

20

NT-3 Analog	Concentration submitted (mg/ml)	Measured concentration (mg/ml)	Percentage of expected activity (%)
Wild-type NT-3	0.32	0.23	72
NT-3$_{(1-117)}$ R61A,K64D	0.36	1.18	328
NT-3$_{(1-119)}$ R61A,K64D	0.25	1.31	524

Both analogs eluted as noncovalent dimers on size
exclusion-HPLC, the same as for wild type NT-3 (see
Figure 5). As expected, there was no significant
25   difference in the molecular weights of the three
proteins. No significant protein aggregation was

detected for either analog. The results of cation
exchange-HPLC (see Figure 6) were consistent with a
reduction in pI for both analogs. The change in
protein charge probably accounts for slight shifts in
5   the SDS-PAGE (Figure 7), compared to wild type NT-3.
Both of the analogs retain the biological activity and
noncovalent dimer structure of wild type NT-3.

Pharmacokinetic Behavior. Serum concentration curves
10   following intravenous administration were seen to be
biphasic (see Figure 8). There was a significant
difference in the initial distribution phase among the
various NT-3 types. The half lifes ($\alpha T_{1/2}$) were 3.3,
5.4 and 7.6 minutes for wild type NT-3, NT-3$_{(1-}$
15   $_{119)}$R61A,K64D, and NT-3$_{(1-117)}$ R61A,K64D, respectively
(see Table 2, below). The observed decrease in
clearance following intravenous administration could be
due solely to the slower distribution of the NT-3
analogs. The most pronounced decrease in concentration
20   during this phase was observed with wild type NT-3.
The terminal phase half lives ($\beta T_{1/2}$) were similar for
the three types and ranged from 0.9 to 1.0 hours,
suggesting that the elimination mechanism for these
types could be the same. The areas under the
25   concentration-time curves (t-AUCinf) for NT-3$_{(1-117)}$
R61A,K64D and NT-3$_{(1-119)}$R61A,K64D were approximately
1.2 to 2-fold that of wild type NT-3.

Table 2. Pharmacokinetic Parameters Obtained in Rats Given a Single IV Dose of 1mg/kg of NT-3 Proteins

NT-3 Type	t-AUC(inf)[1] (ng-hr/ml)	CL[2] (ml/kg-hr)	$\alpha T_{1/2}$[3] (min)	$\beta T_{1/2}$[4] (hr)
Wild type	724.1 ± 151.0	1411.7 ± 294.4	3.3 ± 0.3	1.0 ± 0.6
NT-3(1-119) R61A,K64D	880.7 ± 320.3	1276.2 ± 581.8	5.4 ± 1.9	0.9 ± 0.6
NT-3(1-117) R61A,K64D	1420.7 ± 117.9	706.3 ± 58.7	7.6 ± 0.0	1.0 ± 0.0

1 Area under the serum concentration time curve from time zero to infinity. Area calculation is by trapezoidal method.

2 Clearance rate is calculated by: Dose ÷ AUC.

3 Distribution phase half life ($\alpha T_{1/2}$).

4 Terminal phase half life ($\beta T_{1/2}$).

Following subcutaneous injection, the serum concentration increases rapidly for wild type NT-3 (see Figure 9). The time to maximum concentration (T_{MAX}) was approximately 0.17 hour (see Table 3, below). A slower absorption profile was observed for the modified types. Specifically, T_{MAX}'s for NT-3(1-119) R61A,K64D and NT-3(1-117)R61A,K64D were 0.67 and 1.33 hours, respectively. The maximum concentrations for NT-3(1-119) R61A,K64D and NT-3(1-117)R61A,K64D were 6 to 10 times higher than for wild type NT-3, suggesting that the analogs manifested a greater degree of absorption from the site of injection following administration. Furthermore, the degree or extent of absorption (bioavailability) is more typically determined from the ratio of the areas under the serum curves for subcutaneous and intravenous routes of administration, Table 3. The bioavailability for wild type NT-3, NT-3(1-119) R61A,K64D, and NT-3(1-117)R61A,K64D was 2.2, 28.5 and 43.22%, respectively. The terminal half lifes for the analogs appear to be longer than that of the wild type.

Table 3. Pharmacokinetic Parameters Obtained in Rats
Given a Single SC Dose of 1mg/kg of NT-3 Proteins

NT-3 Type	t-AUC(inf)[1] (ng-hr/ml)	F[2] (%)	CMAX[3] (ng/ml)	TMAX[4] (hr)	$\beta T_{1/2}$[5] (hr)
Wild type	15.2 ± 9.5	2.2 ± 1.3	17.8 ± 11.6	0.2 ± 0.0	0.4 ± 0.0
NT-3(1-119) R61A,K64D	241.3 ± 74.8	28.5 ± 6.3	106.5 ± 47.1	0.7 ± 0.3	1.4 ± 0.6
NT-3(1-117) R61A,K64D	632.9 ± 38.0	43.2 ± 2.0	180.1 ± 51.2	1.3 ± 0.6	1.4 ± 0.8

1 Area under the serum concentration time curve from
  time zero to infinity. Area calculation is by
  trapezoidal method.
2 Bioavailability (F) is calculated by: (t-AUC_{SC} ÷ t-
  AUC_{IV}) x 100%, where both areas were obtained from
  the same animal.
3 CMAX is the maximal concentration.
4 TMAX is the time to maximal concentration.
5 Terminal phase half life ($\beta T_{1/2}$).

     The results from these studies show that by
decreasing the pI of NT-3, one can lower the clearance
rate following intravenous administration, at least
initially, and also enhance the extent of absorption
following subcutaneous administration. These results
also demonstrate that the charge of the protein plays a
significant role in determining pharmacokinetic
behavior. In the case of a basic protein such as NT-3,
as well as other cationic proteins, decreasing the
isoelectric point (or the charge at physiological pH)
can lead to significant improvement in the absorption
and bioavailability of the molecule following
subcutaneous administration. From this knowledge, and
the description provided herein, it is possible to
design new molecules of improved therapeutic value.

     It should be noted that the analogs illustrated in
the foregoing description are intended to be exemplary

only, and that additional analogs of NT-3, as well as of other proteins, can be created in light of the present description to achieve lower isolectric points with longer circulation times and/or higher absorption.
5   In one variation, for instance, the particular analog proteins of SEQ ID NOS: 3 and 5 can be produced by expressed in a mammalian cell or a secreting bacterial strain such that a "met-less" product is obtained (i.e., the methionine residue at the N-terminus is
10   processed away, to result in the polypeptides of SEQ ID NOS: 6 and 7, respectively).

<u>BDNF Analogs</u>

15   Using the procedures described above for NT-3, the following analogs of BDNF were prepared and purified from *E.coli* and the isolectric point (pI) and (approximate) net charge at physiological pH (7.4) were measured for each. The pI and charge at physiological
20   pH for "wild type" BDNF (i.e., of naturally occurring sequence; see U.S. Patent No. 5,180,820, Figure 5) are also shown for purposes of comparison. Positions for substitution are numbered beginning with the first residue following methionine in the mature form of the
25   protein as expressed in *E. coli*. (i.e., the initial methionine residue is not counted).

<u>BDNF, K65D, K73D, K95A, R97A</u>
Substitutions were lysine to aspartic acid at positions
30   65 and 73, lysine to alanine at position 95, and arginine to alanine at position 97.

Calculated pI: 8.46
Charge: +4.0
35

<u>BDNF, P60E, K65D, K73D, K95A</u>

Substitutions were proline to glutamic acid at position 60,

lysine to aspartic acid at positions 65 and 73, and lysine to alanine at position 95.

Calculated pI: 8.45
Charge: +4.0

<u>Wild Type BDNF</u>
Calculated pI: 10.23
Charge: +9.5

<u>Biological Test Results and Characterization</u>

1) <u>In Vitro Bioactivity: Mitogenic Bioassay with PC12/trkC Cells</u>. In this assay, the biological activity of wild type BDNF and the BDNF analogs is determined quantitatively by measuring the incorporation of (MTS) into PC-12 cells (ATCC) that have been transformed to express trkB receptor (the high affinity receptor for BDNF). The transformed cells are maintained at 37 ± 2•C, in a high humidity incubator under an atmosphere containing 7.5 ± 1% carbon dioxide, in Dulbecco's Minimum Essential Medium containing fetal bovine serum and horse serum and 1% L-glutamine. The cells are distributed into well plates for assaying, and incubation is continued. After approximately forty eight hours, the maintenance medium is replaced with RPMI 1640 Medium, the test samples are added, and incubation is continued under the same conditions for another forty eight hours. The cells are then stained with MTS, incubated under the same conditions for another five hours, and the optical density for each well is read with a microplate reader at 490 nm. The

results for bioactivity of the two BDNF analogs versus
wild type BDNF are given in Table 4, below.

Table 4. In Vitro Bioactivity of BDNF Proteins

5

	Bioactivity (mg/mg)
Wild-type BDNF	0.61
BDNF,K65D,K73D, K95A,R97A	1.24
BDNF,P60E,K65D, K73D,K95A	0.61

2) <u>In Vivo Biological Testing and Results</u>. The
biological properties of the analogs were evaluated *in*
vivo in male Sprague Dawley rats at a dose of 3.0
milligrams per kilogram of body weight for BDNF, K65D,
K73D, K95A, R97A and 1.7 milligrams per kilogram of
body weight for BDNF, P60E, K65D, K73D, K95A. The test
materials were administered intravenously and
subcutaneously, followed by collection of blood samples
and measurement of blood serum concentrations (see same
procedure described above for NT-3 and NT-3 analogs).
The pharmacokinetic behavior is given below with
respect to each form of administration.

10

15

20

Table 5. Pharmacokinetic Properties in Rats,
Single Intravenous (IV) Dose of BDNF Proteins

BDNF Type	t-AUC(inf) (ng-hr/ml)
Wild type	8652.8 ± 833.6
BDNF,K65D, K73D,K95A, R97A	12932.8 ± 913.0
BDNF,P60E, K65D,K73D, K95A	14097.2 ± 949.8

5

Table 6. Pharmacokinetic Properties in Rats,
Single Subcutaneous (SC) Dose of BDNF Proteins

BDNF Type	t-AUC(inf) (ng-hr/ml)	F (%)
Wild type	462.5 ± 130.3	4.7 ± 2.0
BDNF,K65D, K73D,K95A, R97A	2401.6 ± 297.1	16.8 ± 0.1
BDNF,P60E, K65D,K73D, K95A	2049.2 ± 357.4	13.9 ± 2.0

10   These results show that the introduction of the
mutations into wild type BDNF has a measurable effect
on pharmacokinetic properties. In particular, greater
in vivo bioavailability is achieved for the two BDNF
analogs in comparison with wild type BDNF as reflected
15   by the increased values for t-AUC(inf) following
intravenous administration and the increase in
t-AUC(inf) and F% following subcutaneous
administration.

20   The invention is defined in the appended claims.